Donation Laboratories

Department of Molecular and Developmental Biology
再生基礎医科学寄付研究部門(SBI, トミー, ロート製薬, 慈照会)

Visiting Professor	Sumiko Watanabe, Ph.D.	特任教授	医学博士　渡　辺　すみ子
Visiting Research Associate	Hideto Koso, MD, Ph.D	特任助教	医学博士　高　祖　秀　登

Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of stem cells as well as differentiated cells with emphasis on intracellular signal transduction. For this purpose we are using models ranging from iPS and various culture cells, zebrafish, mouse, to monkey. Based on our research background on the area of cytokine signals, we now focus on the analysis of development and regeneration of neural retina.
The neural retina is a part of the central nervous system (CNS), and regeneration of the retina from retinal stem cells or other sources by transplantation is a critical issue from both clinical and neurobiological points of view. Although reports of successful regeneration of the CNS from neural stem cells (NSC) have appeared in the literature, such has not been the case for the vertebrate neural retina. Furthermore, the nature of retinal stem cells has not been clarified, making it difficult to attempt regeneration of the retina. Based on the techniques and knowledge that have been accumulated through work on of haematopoietic systems in our laboratory, we attempt to identify mammalian retinal stem cells and following developmental processes by revealing the expression pattern of cell surface proteins. We found that various CD antigens mark spatiotemporally distinct populations of retinal cells, and genes specifically expressed in such populations has been revealed by microarray analyses. Various signaling molecules and transcriptional factors are under investigation for their roles on retinal development. For developmental biological analyses, we use zebrafish in addition to mouse as model animals. We also continue to work on haematological projects, and bidirectional cooperative progress between neurological and haematological works is one of unique features of our laboratory. Projects, which gave major findings during 2012 are as follows.

1. The retinal progenitor-specific gene Sox11 regulates the timing of the differentiation of early-born retinal cells, and its expression is regulated by multiple mechanisms during retinal development

Ayumi Usui[1], Yujin Mochizuki[1], Atsumi Iida, Toshiro Iwagawa, Hiroyuki Aburatani[2], Sumiko Watanabe: [1]Department of Ophthalmology, Juntendo University School of Medicine, [2]Genome Science Division, Research Center for Advanced Science and Technology, University of Tokyo

Sry-related HMG box (Sox) proteins play diverse and critical roles in a variety of morphogenetic processes during embryonic development. Sox11 and Sox4 are members of the SoxC subtype, and we found that Sox11 was strongly expressed in early retinal progenitor cells, and that when expression of Sox11 subsided around birth, Sox4 expres-

sion began. To analyze the role of Sox11 and Sox4 in retinal development, we perturbed their expression pattern by expressing them ectopically in retinal explant culture. Overexpression of Sox11 or Sox4 in retinal progenitors resulted in similar phenotypes, that is, increased cone cells and decreased Müller glia. Sox11-knockout retinas showed delayed onset and progress of differentiation of early-born retinal cells during the embryonic period. After birth, retinal differentiation took place relatively normally, probably because of the redundant activity of Sox4, which starts to differentiate around birth. Neither overexpression nor loss-of-function analysis gave any evidence that Sox11 and Sox4 directly regulate transcription of genes critical to early-born retinal cells. However, histone H3 acetylation status of the early neurogenic genes was lowered in knockout retinas, suggesting that Sox11 regulates the timing of differentiation in early-born retinas by creating an epigenetic state that helps to establish the competency to differentiate. We also examined molecular basis of regulation of expression of Sox11 and Sox4. Gain-of-function and loss-of-function analyses suggested that Notch signal suppresses Sox11 expression in the early developing retina but not during the later period of development. The levels of histone H3-acetylation and H3-lysine 4 tri-methylation at the *Sox4* and *Sox11* loci were dramatically changed, as were the levels of Sox4 and Sox11. In contrast, negative regulation of histone H3-lysine 27 methylation was observed, with reciprocal alterations to the levels of Sox4 and Sox11.

2. Differential regulation of expression of the long/middle wavelength-sensitive opsins of vertebrates is mediated by thyroid hormone receptor β2 and COUP-TFII

Toshiro Iwagawa, Yo Tanaka, Atsumi Iida, Sumiko Watanabe

Cone photopigments (opsins) are crucial elements of, and the first detection module in, color vision. Individual opsins have different wavelength sensitivity patterns, and the temporal and spatial expression patterns of opsins are unique and stringently regulated. Long and middle wavelength-sensitive (L/M) opsins are of the same phylogenetic type. Although the roles of thyroid hormone and TRβ2 in the transcriptional regulation of L/M opsins have been explored, the detailed mechanisms, including the target sequence in the enhancer of L/M opsins, have not been revealed. Using several human red opsin enhancer/promoter-luciferase reporter constructs, we found that TRβ2 increased luciferase activities through the 5'-UTR and intron 3-4 region, whereas the presence of T3 affected only the intron 3-4 region-dependent luciferase activity. Furthermore, COUP-TFII suppressed intron 3-4 region-dependent luciferase activities. However, luciferase expression driven by the mouse M opsin intron 3-4 region was only slightly increased by TRβ2, and rather enhanced by COUP-TFII. To determine whether these differential responses reflect differences between primates and rodents, we examined the enhancer/promoter region of the red opsin of the common marmoset. Interestingly, while TRβ2 increased 5'-UTR- or intron 3-4 region-driven luciferase expression, as observed for the human red opsin, expression of the latter luciferase was not suppressed by COUP-TFII. In fact, immunostaining of common marmoset retinal sections revealed co-expression of COUP-TFII and red opsin in the cone cells. These results suggest species-specific differential regulation of L/M opsins by TRβ2 and COUP-TFII.

3. Use of cell type-specific transcritome to identify genes specifically involved in Müller glia differentiation during retinal development

Yujin Mochizuki[1], Atsumi Iida, Akira Murakami[1], Sumiko Watanabe

Retinal progenitor cells alter their properties over the course of development, and sequentially produce different subpopulations of retinal cells. We had previously found that early and late retinal progenitor cell populations can be distinguished by their surface antigens, SSEA-1 and c-kit, respectively. Using DNA microarray analysis, we examined the transcriptomes of SSEA-1 positive cells at E14, and c-kit positive and c-kit negative cells at P1. By comparing data, we identified genes specifically expressed in c-kit positive late retinal progenitor cells. The previous literature suggests that most of the c-kit positive cell-specific genes are related to glia differentiation in brain or are expressed in Müller glia. Since Notch signaling promotes Müller glia differentiation in retina, we examined the effects of gain- and loss-of-Notch signaling on expression of these genes and found that all the genes were positively affected by Notch signaling. Finally, we screened the genes for their function in retinal development by shRNA-based suppression in retinal explants. In about half the genes, Müller glia differentiation was perturbed when their expression was suppressed. Taken together, these results show that at P1, c-kit positive retinal progenitor cells, which include Müller glia precursor cells, are enriched for genes related to glial differentiation. We propose analysis of purified subsets of retinal cells as a powerful tool to elucidate the molecular basis of retinal development.

4. Molecular mechanisms of brain tumor initiation

Hideto Koso, Eli Lyons, Asano Tsuhako, Sumiko Watanabe

NSCs are considered to be the cell of origin of glioblastoma multiforme (GBM). However, the genetic alterations that transform NSCs into glioma-initiating cells remain elusive. Using a novel transposon mutagenesis strategy that mutagenizes NSCs in culture, followed by additional rounds of mutagenesis to generate tumors *in vivo,* we have identified genes and signaling pathways that can transform NSCs into glioma-initiating cells. Mobilization of *Sleeping Beauty* transposons in NSCs induced the immortalization of astroglial-like cells, which were then able to generate tumors with characteristics of the mesenchymal subtype of GBM upon transplantation, consistent with a potential astroglial origin for mesenchymal GBM. Sequence analysis of transposon insertion sites from tumors and immortalized cells identified more than two hundred frequently mutated genes, including human GBM-associated genes such as *Met* and *Nf1*, and made it possible to discriminate between genes that function during astroglial immortalization versus later stages of tumor development. This novel mutagenesis strategy is faster and simpler than conventional transposon screens and can potentially be applied to any tissue stem/progenitor cells that can be grown and differentiated *in vitro*.

5. Temporal regulation of Cre activity in NSCs and rod photoreceptors

Hideto Koso, Asano Tsuhako, Sumiko Watanabe

The RNA-binding protein Musashi1 (Msi1) is one of two mammalian homologues of *Drosophila* Musashi, which is required for the asymmetric cell division of sensory organ precursor cells. In the mouse central nervous system, Msi1 is preferentially expressed in mitotically active NSCs in the ventricular zone of the neural tube during embryonic development and in the subventricular zone (SVZ) of the postnatal brain. We described the generation of *Msi1-CreER^{T2}* knock-in mice and showed by cell lineage tracing that *Msi1-CreER^{T2}*-expressing cells specifically mark NSCs in both the embryonic and adult brain. We also showed that *Msi1-CreER^{T2}* is expressed in photoreceptor cells of the mature retina. *Msi1-CreER^{T2}* mice thus represent a new tool in our arsenal for genetically manipulating NSCs and photoreceptor cells, which will be essential for understanding the molecular mechanisms underlying diseases of the nervous system.

6. Molecular mechanisms regulating differentiation and proliferation of retinal stem/progenitor cells: Requirement of Fezf2 in Differentiation of Cone OFF bipolar cells

Haruna Suzuki-Kerr, Sumiko Watanabe

Cell surface antigens are powerful tools for isolating specific subsets of retinal cells during development from cell mixtures without damaging the cells, which makes it possible to characterize their properties and identify genes that regulate their proliferation and differentiation. By screening retinal cells from mice at various developmental stages for their reactivity with over 150 different antibodies against various cell surface antigens, we identified SSEA-1 and c-kit as early and late progenitor markers, respectively. SSEA-1 marks retinal progenitor cells in the peripheral region of the retina at around E14-E16. In the later stage of embryogenesis, SSEA-1 disappears and c-kit expression is observed in the retinal progenitor cells in the central region of the retina. We compared the gene expression patterns of regionally and temporally different subsets of retinal progenitor cells, SSEA-1-positive cells at E14, c-kit positive cells at P1, and differentiated c-kit negative cells at P1 using a microarray. We found that several genes are specifically expressed in SSEA-1 positive early retinal progenitor cells. Currently, we are focusing on one of such genes, Fezf2. We found that the Fezf2, a transcriptional repressor of the Fez zing finger family, to be strongly expressed in SSEA-1-positive cells. Subsequent analysis showed that Fezf2 was expressed in developing bipolar cells and that there was a significant reduction in the number of OFF type bipolar cells in the Fezf2KO retina, suggesting a role played by Fezf2 during bipolar cell differentiation/maturation. We further examined the role of Fezf2 in bipolar differentiation by Electroretinogram (ERG), commonly used to assess the functionality of the retina *in vivo*. In 2-month-old Fezf2KO retina, cone ON bipolar response was significantly reduced, suggesting that these cells are not fully functional in Fezf2KO retina. We then examined the synaptic structure of ON bipolar cells by transmission electron microscopy (TEM). Ultra-structural analysis by TEM revealed that the degree of contact between ON bipolar dendrite and cone photoreceptor terminal was slightly, but significantly reduced in Fezf2KO retina. Since Fezf2 was not expressed in ON bipolar cells, these changes to cone ON bipolar cells in Fezf2KO retina may represent an interaction between cone OFF and ON bipolar cells during maturation of bipolar cells. Next, we analyzed the time course of Fezf2 expression, and found that Fezf2 expression increased in a subset of retinal bipolar cells at around P8, around the time of bipolar maturation. As there are at least 10 morphologically distinct subtypes of bipolar cells in the mouse retina, we then investigated the expression of bipolar subtype specific markers. So far, the use of subtype-specific markers for IHC & qPCR suggests that type III OFF bipolar subtype marker is down regulated

in Fezf2KO. Taken together, our results suggest that Fezf2 may be involved in OFF bipolar subtype specification during development of the retina. The current finding should help clarify molecular mechanisms behind differentiation of retinal progenitors and their subsequent maturation into a specific subtype of neurons.

7. Nucleolar phosphoprotein NPM1 regulates Vsx2 gene expression in retinal progenitors by binding to evolutionarily conserved enhancer elements

Yasuo Ouchi, Yukihiro Baba, Sumiko Watanabe

The homeodomain transcription factor Vsx2 is the earliest characterized specific marker of retinal progenitor cells and is known to play an important role in their proliferation. To better understand the evolutionarily conserved molecular mechanism of retinal progenitor cell maintenance, we attempted to identify Vsx2 promoter and upstream regulators using zebrafish and mice. By database analysis of Vsx2 loci, we identified four evolutionarily conserved sequences in the Vsx2 loci. Through a series of transient expression assays conducted with EGFP reporter plasmids, we identified a *cis*-regulatory motif located 35 kb upstream of the mouse Vsx2 gene as an evolutionarily conserved enhancer in retinal progenitor cells. A proteomic analysis conducted using a minimal *cis*-regulatory motif identified proteins, including NPM1, that bind to this region. The mouse and zebrafish homologs of NPM1 were shown to be strongly expressed in retinal progenitor cells during development. NPM1 enhanced Vsx2 enhancer-luciferase reporter gene expression, and the C-terminal DNA-binding motif of NPM1 was essential for this activity. Moreover, downregulation of NPM1 expression by shRNA reduced the number of Vsx2-positive cells, inhibited cell proliferation, and induced apoptosis in retinal progenitor cells. Collectively, these results reveal that NPM1 plays a critical role in retinal development by regulating Vsx2 gene expression.

8. In Vitro Cell subtype-specific transduction of adeno-associated virus in the mouse and marmoset retinal explant culture

Yukihiro Baba, Sumiko Watanabe

Adeno-associated virus (AAV) is a non-pathogenic human parvovirus that can infect both non-proliferating and proliferating cells. Owing to its favorable safety profile, AAV is regarded as suitable for clinical purposes such as gene therapy. The target cell types of AAV depend largely on the serotype. In the retina, AAV has been used to introduce exogenous genes into photoreceptors, and photoreceptor-specific enhancers/promoters are used in most cases. Therefore, serotype specificity of AAV in retinal subtypes is unclear, particularly *in vitro*. We compared its infection profile in mouse and monkey retinas using EGFP under the control of the CAG promoter, which expressed the gene ubiquitously and strongly regardless of cell type. AAV1, 8, and 9 infected the horizontal cells when an embryonic day-17 retina was used as a host. Nearly 100% of the horizontal cells expressed EGFP. Amacrine cells were also a target of AAVs, and a small number of rod photoreceptors were infected. When adult retinas were used as a host, the main target of AAV was Müller glia. A small number of rod photoreceptors were also infected. In the adult common marmoset retina, rod and cone photoreceptors were efficiently infected by AAV1, 8, and 9. A portion of the Müller glia and amacrine cells were also infected. In summary, the infection specificity of different AAV serotypes did not differ, but was dependent on the stage of the host retina. In addition, infection specificities differed between mature marmoset retinas and mature mouse retinas.

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Donation Laboratories

Division of Antibody, Vaccine & Experimental Therapy
抗体・ワクチン治療寄付研究部門

Project Associate Professor	Taniguchi Hiroaki, M.D., D.M.Sc.	特任准教授	医学博士	谷口博昭
Professor	Motoharu Seiki, D.M.Sc.	教授(兼)	医学博士	清木元治
Professor	Kohzoh Imai, M.D., D.M.Sc.	教授(兼)	医学博士	今井浩三
Professor	Kouhei Tsumoto, Ph.D.	教授(兼)	工学博士	津本浩平

Tumors contain a small population of putative cancer stem cells (CSC), which possess unique self-renewal properties, and survive in a quiescent state for many years after remission and result in later relapse and metastasis. Therefore, it is conceivable that targeting CSCs will eradicate tumor-initiating cells, whereas conventional chemotherapies will only eradicate the bulk of a tumor.
Cancer stem cells and normal tissue stem cells utilize the same self-renewal pathway. However, researchers characterize some of changes, which occur in cancer stem cells, not in normal tissue stem cells. The design of new therapeutic agents should be aimed at targeting these unique molecular changes.
We have currently focused on studying these unique molecular changes, which occur in cancer stem cells, not in normal tissue stem cells. This could be a new therapeutic target against solid tumors.

A) Zinc-finger-containing transcriptional factor, Kruppel-like factor 2 (KLF2)

The Kruppel-like factor (KLF) proteins are multitasked transcriptional regulators with an expanding tumor suppressor function. KLF2 is a member of the KLF family of zinc-finger transcription factors and is involved in maintaining T-cell quiescence, regulating preadipocyte differentiation, endothelial cell function, lung development and the self-renewal of ES cells. Furthermore, KLF2 is one of the prominent members of the family because of its diminished expression in malignancies and its growth-inhibitory, pro-apoptotic and anti-angiogenic roles.

We indicate that epigenetic silencing of KLF2 occurs in cancer cells through direct transcriptional repression mediated by the Polycomb group protein Enhancer of Zeste Homolog 2 (EZH2). Binding of EZH2 to the 5'-end of KLF2 is also associated with a gain of trimethylated lysine 27 histone H3 and a depletion of phosphorylated serine 2 of RNA polymerase.

Upon depletion of EZH2 by RNA interference, short hairpin RNA or use of the small molecule 3-Deazaneplanocin A, the expression of KLF2 is restored. The transfection of KLF2 in cells with EZH2-associated silencing showed a significant anti-tumoral effect, both in culture and in xenografted nude mice.

In this last setting, KLF2 transfection was also associated with decreased dissemination and lower mortality rate. In EZH2-depleted cells, which characteristically have lower tumorigenicity, the induction of KLF2 depletion `rescued' partially the oncogenic phenotype, suggesting that KLF2 repression has an important role in EZH2 oncogenesis.

Most importantly, the translation of the described results to human primary samples demonstrated that patients with prostate or breast tumors with low levels of KLF2 and high expression of EZH2 had a shorter overall survival.

B) PR domain-containing protein, PRDM14

PRDM have been linked to human cancers. To explore the role of the PR domain family genes in breast carcinogenesis, we examined the expression profiles of 16 members of the PRDM gene family in a panel of breast cancer cell lines and primary breast cancer specimens using semiquantitative real-time PCR.

We found that PRDM14 mRNA is overexpressed in about two thirds of breast cancers. Moreover, immunohistochemical analysis showed that expression of PRDM14 protein is also up-regulated. PRDM14 are known as a key transcription factor required for the maintenance of hESC identity and the reacquisition of pluripotency in human somatic cells.

Introduction of PRDM14 into cancer cells reduced their sensitivity to chemotherapeutic drugs. Conversely, knockdown of PRDM14 by siRNA induced apoptosis in breast cancer cells and increased their sensitivity to chemotherapeutic drugs. Moreover, PRDM14 regulated cancer metastasis, angiogenesis, and stemness of cancer cells.

That little or no expression of PRDM14 is seen in noncancerous tissues suggests that PRDM14 could be an ideal therapeutic target for the treatment of breast cancer. Now, we also develop new methodology with nuclear acid medicine and modified antibody drug against PRDM14.

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Project Associate Professor, Taniguchi Hiroaki:

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Professor, Motoharu Seiki:

Please refer to Division of Cancer Cell Research, Department of Cancer Biology.

Professor, Kohzoh Imai:

Please refer to Center for Antibody and Vaccine Therapy.

Professor, Kouhei Tsumoto:

Please refer to Medical Proteomics Laboratory.

Donation Laboratories

Division of Social Communication System for Advanced Clinical Research

先端医療社会コミュニケーションシステム社会連携研究部門

Project Professor Masahiro Kami, M.D., Ph.D.
Project Assistant Professor Tomoko Matsumura, M.D., Ph.D.

特任教授 医学博士 上 昌広
特任助教 医学博士 松村有子

The aim of our division is to establish and popularize state-of-art medicine and to promote translational research (TR). We investigate medical governance and the methodology to develop national consensus in health care by using media.
We also perform individual case studies on economic burden of health care on patients, medical support for disaster-stricken area by the Great East Japan Earthquake on March 11, 2011 and physician supply. In each case, we also study the system of management, information circulation, and network.

【Medical Governance】

Tomoko Matsumura, Naoko Murashige, Yuko Kodama, Koichiro Yuji[1], Masaharu Tsubokura, Masahiro Kami, Tetsuya Tanimoto[2], Shuichi Iwamoto[2], Masayoshi Nagata[3]: [1]Department of Medicine (Department of Hematology / Oncology), Institute of Medical Science, the University of Tokyo, [2]Navitas Clinic, Internal Medicine, [3]Department of Urology, the University of Tokyo

We performed mathematical simulation of changes in age/sex composition of the population, fatalities and the number of physicians in the future to predict the impact of the current government plan to increase medical school enrollments. In addition, we also studied the effect of genetic regulations on clinical practice, the system of management, quality-of-life effects of screening, drug approval system by the regulatory agency, Japan's vaccination policy, information circulation and published the results in scientific journals (Yuji K, et al., Murashige N, et al., Nagata M, et al., Tanimoto T, et al., Iwamoto S, et al. Tanimoto T, et al.). These results were widely published in news papers and popular magazines.

【Medical support for disaster-stricken area】

Masaharu Tsubokura, Yukio Kanazawa[3], Tomoyoshi Oikawa[3], Kyohei Takahahi[4], Akemi Takada[3], Tomoko Matsumura, Morihito Takita, Tamae Hamaki[2], Kazuhiko Kobayashi[5], Syuichi Iwamoto[2], Ginichi Mori[6], Yukie Takahashi[6], Masaki Miyasaka[7], Hideki Komatsu[8], Makoto Suzuki[8], Mamiko Ohara[8], Tsunehiko Komatsu[9], Kenji Shibuya[10], Amina Sugimoto[10], Syuhei Nomura[10], Tetsuya Tanimoto[2], Ken Okai[3],Takeaki Ishii[11], Shigeaki Kato[11], Sae Ochi[11], Giichiro Oiso[12], Yuko Kodama, Masahiro Kami: [3]Minamisoma Municipal General Hospital, [4]Haramachi Central Maternity Clinic, [5]JR Tokyo General Hospital, [6]Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital, [7]Tokyo Metropolitan Bokutoh Hospital, [8]Kameda Medical Center, [9]Teikyo University Chiba Medical Center, [10]Pharmaceuticals and Medical Devices Agency, Graduate school of Medicine, Department of Global Health Policy, The University of Tokyo, [11]Soma Central Hospital,

Collaborating with physicians who work in disaster-stricken area and many support physicians, we conducted measurement of internal radiation exposure and gave medical guidance to the local people and publish the results in scientific journals (Tsubokura M, et al., Tanimoto T, et al. Harasawa K, et al., Tsubokura M, et al., Okai K, et al., Ishii T, et al., Tsubokura M, et al). These results were widely published in news papers and popular magazines.

【Economic Burden of Health Care on Patients】

Yuko Kodama, Ryoko Morozumi[13], Akihiko Matsui[14], Masahiro Kami, Tomoko Matsumura, Naoko Murashige, Morihito Takita, Eiji Kusumi[2]: [13]Faculty of Economics, University of Toyama, [14]Faculty of Economics, the University of Tokyo

Imatinib (Glivec), which is the first-line drug for chronic myelogenous leukemia (CML), is highly efficient. We clarified that the cost of Glivec in Japan was higher than the other countries by international research. We conduct a collaborate study with Professor Matsui at Faculty of Economics, the University of Tokyo, to review the utilization of Glivec and its cost in Japan. Because the economic burden on patients or the government with prevailing advanced medical care including anticancer drugs is an important issue, we continue further investigation. (Kodama Y, et al.)

【Clinically Oriented Research】

Tomoko Matsumura, Tetsuya Tanimoto[2], Hiroto Narimatsu[15], Natsuko Watanabe[16] Ginichi Mori[6], Yukie Takahashi[6], Masaki Miyasaka[7]: [15]Graduate School of Medicine / Cohort Management Unit, Yamagata University, [16]Ito Hospital

We performed a retrospective cohort study involving more than 50 thousand patients with Graves' disease and a retrospective survey involving more than 250 patients with acute lymphoblastic leukemia. We also studied in prophylaxis and treatment in lymphoma. These results were published in scientific journals (Watanabe N, et al., Matsumura T, et al., Tanimoto T, et al., Mori J, et al., Takahashi Y, et al.).

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Interactome Medical Sciences Laboratory
インタラクトーム医科学社会連携研究部門

Project Associate Professor Etsuko Miyamoto-Sato, Ph.D. | 特任准教授 工学博士 宮 本 悦 子

In our lab, the interactome (protein-protein interactions) is analyzed using technology developed in Japan and referred to as "puromycin technology". Toward the "personalized medicine" era, we will apply these tools to medical science. To develop the "interactome analysis pipeline", we are collaborating with the Human Genome Center, where large amounts of sequence data are generated from next-generation sequencers and analyzed using a supercomputer. In particular, our research focuses on "cancer", and we collaborate with cancer researchers. For a cooperative study involving society and academia, our research involves industrial partnerships.

The dynamic whole-cell omics analyses to understand and control cancer stem cells

Shigeo Fujimori[1], Takatsune Shimizu[1,2], Rui Yamaguchi[3], Hiroyuki Ohashi[1], Seiya Imoto[3], Naoya Hirai[1], Yukiko Kawabata[1], Hideyuki Saya[2], Satoru Miyano[3], Etsuko Miyamoto-Sato[1,3]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. of Tokyo [2]Div. Gene Reg., IAMR, Keio Univ. Sch. Med. [3]Human Genome Ctr., Inst. Med. Sci., Univ. of Tokyo

Recent scientific discoveries resulted from the next-generation sequencing (NGS) highlight the striking impact of massively parallel sequencing data on genetics. Thus, the interest of basic research in the medical care has changed from the conventional post-genomic to the personal genomic research, especially for cancer therapy. To understand the individuality of cancer, we have to conduct various dynamic omics analyses and comprehend the individual biomolecular networks for each cell type. Our research focuses on diversity of cancer stem cells (CSCs), and is aimed at discerning and regulation of CSCs by the collaboration of researchers, who have the experiment system for studying CSCs, the technology for omics analysis, and the network analysis technique. We have model CSCs derived from bone-marrow stromal cells of Ink4a/Arf KO mice (Shimizu et al., *Oncogene*, 2010). To compare those CSCs having distinct characters, we attempt to conduct "dynamic whole-cell omics" analyses, especially for interactome sequencing. To collect comprehensive protein level's data from NGS, we have developed IVV-HiTSeq (Fujimori et al., *Scientific Reports*, 2012) standing for the *in vitro* virus method (Miyamoto-Sato et al., *PLoS ONE*, 2010) coupled with High-Throughput Sequencing. IVV-HiTSeq can obtain reliable interactome data suitable for the medical field due to low false positives. Moreover, we attempt to develop the bait-free IVV method as IVV square (IVV^2) to obtain not partial but a whole-cell interactome. IVV^2 also detects 'interacting regions (IR)', allowing us to analyze relations between interactome and aberrance (e.g., DNA mutation) observed in cancer. Accordingly, we have developed the database to investigate such relations.

IRView: a database and viewer for protein interacting regions

Shigeo Fujimori[1], Naoya Hirai[1], Tomohiro Oshikubo[1,2], Tatsuhiro Yamashita[1,3], Takanori Washio[1,4],

Ayumu Saito[5], Satoru Miyano[5], Etsuko Miyamoto-Sato[1,5]: [1]Div. Interactome Med. Sci., Inst. of Med. Sci., Univ. of Tokyo [2]FAE Ltd. [3]Fujitsu Ltd. [4]RIKEN GENESIS Co., Ltd. [5]Human Genome Ctr, Inst. of Med. Sci., Univ. of Tokyo

Protein-protein interactions (PPIs) and their networks play central roles in governing cellular processes. In recent years, the interest of researchers has shifted from the conventional genomics to the personal genomics, bringing a new challenge to examine the impact of small differences in genomes among individuals, cells and tissues on the interactomes. Although basic relationships between proteins have been compiled in public databases at the protein level, proteins interact with other proteins through regions (i.e. the interacting regions; IRs) specific to each interactant, thereby simultaneous interactions with multiple proteins become possible. Furthermore, some IRs are competitively used for different proteins. Therefore, to comprehend such complex relations underlying protein interactions and to reveal differences of networks among individuals further refined interaction data are needed. The IRView (http://ir.hgc.jp/) is our developed database for IRs, which focuses on regions required for PPIs (Fujimori et al, *Bioinformatics* 2012). IRView currently contains data for over 3,000 IRs determined mainly with mRNA-display, known as the *in vitro* virus (IVV) method (Miyamoto-Sato et al, *PLoS ONE* 2010). The IVV method employs the cDNA library created by the random priming RT-PCR for poly(A) + RNA library for screening, allowing us to detect not only interacting proteins with bait, but also their regions. The IR data stored in the IRView are combined with annotated region data such as InterPro, SNPs and variable regions owing to alternative mRNA splicing.

PRD: A protein-RNA interaction database

Shigeo Fujimori[1], Katsuya Hino[2], Ayumu Saito[3], Satoru Miyano[3], Etsuko Miyamoto-Sato[1,3]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. of Tokyo [2]Dept. Biosci. & Info., Keio Univ. [3]Human Genome Ctr, Inst. of Med. Sci., Univ. of Tokyo

Although protein-RNA interactions (PRIs) are involved in various important cellular processes, compiled data on PRIs are still limited. This contrasts with protein-protein interactions, which have been intensively recorded in public databases and subjected to network level analysis. We developed PRD, an online database of PRIs, dispersed across several sources, including scientific literature. Currently, over 10,000 interactions have been stored in PRD using PSI-MI 2.5, which is a standard model for describing detailed molecular interactions, with an emphasis on gene level data. Users can browse all recorded interactions and execute flexible keyword searches against the database via a web interface. Our database is not only a reference of PRIs, but will also be a valuable resource for studying characteristics of PRI networks. AVAILABILITY: PRD can be freely accessed at http://pri.hgc.jp/

A whole-cell interactome mapping using IVV square toward personal genomics

Hiroyuki Ohashi[1], Shigeo Fujimori[1], Naoya Hirai[1], Takatsune Shimizu[2], Hideyuki Saya[2] and Etsuko Miyamoto-Sato[1]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. of Tokyo [2]Div. Gene Reg., IAMR, Keio Univ. Sch. Med.

In the last decade, huge amount of genomic sequence data have been determined and gathered. The Human Genome Project provided the human genome sequence. Understanding the genetic sequences of individual patients is becoming a central feature of medical care. It is very important that the accumulation of genetic alterations and the interactome networks in cancer cells represents a universal feature of the disease. The knowledge can contribute to determining optimal therapy for the disease. Since protein-protein interactions (PPIs) are at the core of the biomolecule network, we have developed *in vitro virus* (IVV) system, which is a mRNA display, to detect PPIs toward personal genomics. We have succeeded to obtain significant results of interactome analysis by using the system. On the other hand, the system has a limitation in the high-throughput screening and identification of interaction pairs of proteins, due to the time consuming preparation of bait proteins and the low ability of the conventional sequencing method. To overcome the problem, we are trying to develop "a bait-free IVV", termed IVV Square (IVV^2), which enables genes encoding interacting protein pairs to be linked. This will facilitate archiving of the interactome mapping of a whole-cell library. IVV^2 libraries will be subjected to the high-throughput sequencing with the next-generation sequencers to generate interactome information. We believe that the new system will contribute to understand the interactome networks in cancer cells, and to develop pharmaceutical drugs to treat intractable diseases.

Efficiency of puromycin-based technologies mediated by release factors and a ribosome recycling factor

Hiroyuki Ohashi[1], Naoya Hirai[1] and Etsuko Miyamoto-Sato[1]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. of Tokyo

Two useful puromycin-based techniques, *in vitro* virus (IVV) and C-terminal labelling of proteins,

were developed based on the observation that puromycin can bind to the C-terminus of a full-length protein. Puromycin technology is a useful tool for the detection of proteins and analysis of protein-protein interactions (PPIs); however, problems arise due to the existence of stop codons in the native mRNAs. Release factors (RFs) enter the A site of the ribosome at stop codons, which inevitably compete with the puromycin. To overcome this difficulty, we have used a highly controllable reconstituted cell-free system for puromycin-based techniques and observed efficient IVV formation and C-terminal labelling using templates possessing a stop codon. The optimal conditions of IVV formation using templates possessing a stop codon was RF (−), while that of C-terminal labelling was RF (−) and the ribosome recycling factor (RRF) (+). Thus, we have overcome the experimental limitations of conventional IVV. In addition, we discovered that RRF significantly increases the efficiency of C-terminal protein labelling, but not IVV formation.

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Donatinon Laboratories

Division of RNA Medical Science
「RNA医科学」社会連携研究部門

Project Assoiate Professor	Akira Ishiguro, Ph.D.	特任准教授	理学博士　石黒　亮
Project Assistant Professor	Takahiro Negishi, Ph.D.	特任助教	薬学博士　根岸崇大

Protein non-cording RNAs can be classified into two types: one, like antisense and microRNA, those function with the sequence complementarity to the target mRNA or DNA, while the other, like aptamer, those function independent of the sequence complementarity.
RNA aptamer is a biochemical or therapeutic agent that targets a given molecule that is selected by a process known as SELEX (systematic evolution of ligands by exponential enrichment) from a complex library of random RNA sequences of typically 10^{14} different molecules. The concept is based on the ability of short (20-80 mer) sequences to fold, in the presence of a target, into unique three-dimensional structures that bind the target with high affinity and specificity. This binding, in many cases, leads to a blockade of protein activity. Therefore, aptamers can be thought of as nucleic acid analogs to antibodies.
By studying RNA aptamers, we hope to clarify superior potential of RNA, which would be highly beneficial to the development of RNA medicine and the comprehensive understanding of RNA and protein functions.

1. Therapeutic Development of RNA Aptamer against Interleukin-17

Akira Ishiguro, Shigeyuki Mori[1], Natsuki Otaki[1], Haruta Kazuhiko[1], Shoichiro Shibata, Michiru Ozawa, Tomoyo Kimijima[1], and Yoshikazu Nakamura.: [1]Zenyaku Kogyo Co. Ltd.

Interleukin-17A (IL-17A) is a pro-inflammatory cytokine produced primarily by a subset of CD4 + T cells, called Th17 cells, that is involved in host defense, inflammation and autoimmune disorders. We have reported previously the successful isolation of RNA aptamer against IL-17A, named Apt21-2, and its antagonistic activity against the in vitro and in vivo functionality of IL-17A, implicating its potent therapeutic potential (Ishiguro et al. 2011). Toward clinical application, we improved the activity and pharmacokinetic property of Apt21-2 by ribose 2' modifications and 5' PEGylation or a conjugation of inverted dT. Through screening more than 300 chemically synthesized derivatives of Apt21-2, the best candidate showed the IC_{50} value 40 fold smaller than that of Apt21-2 in the cell-based in vitro assay. We trust that this candidate aptamer should be applicable to the clinical trial after toxicity test.

2. Selection of RNA Aptamer against Fibroblast Growth Factor 2 and its Therapeutic Application

Akira Ishiguro, Maiko Sakamoto, Michiru Ozawa, Shoichiro Shibata, Yoshikazu Nakamura

Fibroblast growth factor is a class of heparin binding proteins that mediate a variety of cellular responses during embryonic development and in the adult organism. Fibroblast growth factor 2 (FGF2 or bFGF for basic fibroblast growth factor)

has an important role in proliferation, migration, angiogenesis and morphogenesis. Secreted FGF2 protein activates several cell signaling such as JNK, MAPK, via through FGF receptors on the cell surface. Recent reports suggested that enhanced expression in human RA patients, and also elevated in rat joints of adjuvant-induced arthritis (AIA).

By screening of a large library of nuclease-resistant RNA oligonucleotides by SELEX, we selected an RNA aptamer that bind human and mouse FGF2 proteins with high affinity and specificity. The selected aptamer binds strongly to FGF2, but not to FGF1, and inhibits the interaction between FGF2 and its receptor when examined by the SPR (surface plasmon resonance) analysis. Consistently, the aptamer prevented efficient phosphorylation of FGF2 signaling factors, FRS2 and MAPK, in the cell-based assay with NIH3T3. Furthermore, the aptamer inhibited the FGF2-dependent repression of OPG (osteoprotegerin) secretion in HFLS-RA (Human Fibroblast-Like Synoviocytes cells from RA patient).

We then demonstrated that *in vivo* efficacy of the aptamer using GPI-induced rheumatoid arthritis mice model. When administrated immediately after immunization with GPI, the aptamer inhibited the development of arthritic symptoms in a dose-dependent manner. Significantly, the aptamer slowed the progression of arthritis when administered after the onset of GPI induced arthritis. Our findings indicate that the chemically processed anti-FGF2 aptamer inhibits FGF2 action and the development of RA in mouse models. These results offer, for the first time an aptamer-based therapeutic approach for FGF2 related disorders.

3. Selection of Aptamer Antagonists against Mouse Shh Protein and its Application as a Novel Silencing Tool in Developmental Biology

Akira Ishiguro

We aim to apply aptamer technology to the development of a novel protein-silencing tool, which is applicable to dissection and/or discovery of the role of protein of interest in developmental biology. As a first model case, we selected RNA aptamers that specifically inhibit mouse sonic hedgehog protein, mShh. S9 trim-3 is one such aptamer raised against mShh protein, specifically binds to mShh with high affinity.

During vertebrate development, morphogen gradients generate different cell types in distinct spatial order. Secreted proteins of this class, such as HH, BMP, Wnt, FGF family proteins, play essential roles in normal cell differentiation, axis determination and patterning many systems. Shh is a well established morfogen, and plays various roles in development such as, patterning the central nervous system, during limb development, and formation of facial structures. The Shh signaling pathway involves two transmembrane proteins, Patched (Ptc) and Smoothened (Smo): Shh binds Ptc, whereas Smo acts as a signal transducer.

In this program, aptamers were selected against mShh by SELEX from RNA pools randomized over 30 nucleotides with 2'-fluoro pyrimidine modifications to resist ribonucleases. Isolated aptamer candidates were examined for the binding affinity to mShh protein by the SPR assay. The highest affinity of the aptamer to mShh showed the dissociation constant 15nM. The mShh protein has been shown to control osteoblast differentiation in vertebrates. The differentiated C3H10T1/2 embryonic fibroblast cells produce vascular endothelial growth factor (VEGF). The selected aptamer, S9 trim-3, inhibited the mShh-dependent differentiation of C3H10T1/2 cells and blocked secretion of VEGF. Thus, the in vivo efficacy of anti-mShh aptamer remains to be investigated in mouse model.

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Division of Bacterial Infection Biology

細菌感染生物学社会連携研究部門(ヤクルト本社, 日本生物化学研究所)

Project Associate Professor	Minsoo Kim, Ph.D.	特任准教授 理学博士	金　玟秀
Project Assistant Professor	Hiroshi Ashida, Ph.D.	特任助教 医学博士	芦田　浩

Many pathogenic bacteria, including Shigella, enteropathogenic Escherichia coli (EPEC), and enterohemorrhagic E.coli (EHEC), are associated with diarrheal diseases and are an important cause of death in many countries. Our current interest is to understand the complex interactions among pathogenic bacteria, the gastrointestinal epithelium and microbiota during pathogenic bacteria infection. The main goal of our research is to develop new therapeutic tools or vaccines that will target these bacterial infections.

1. A bacterial effector deamidates Ubc13 to dampen the inflammatory response.

Sanada T, Kim M, Mimuro H, Suzuki M, Ogawa M, Oyama A, Ashida H, Kobyashi T, Koyama T[1], Nagai S[1], Shibata Y[2], Gohda J[2], Inoue J[2], Mizushima T[3], and Sasakawa C.: [1]Nippon Institute for Biological Science, Shinmachi, Ome, Tokyo, Japan, [2]Division of Cellular and Molecular Biology; Department of Cancer Biology; Institute of Medical Science, University of Tokyo, Tokyo, Japan, [3]Picobiology Institute; Department of Life Science; Graduate School of Life Science; University of Hyogo, Hyogo, Japan.

Upon infection of many bacterial pathogens, bacterial invasion is quickly sensed by the innate immune system and triggers acute inflammatory responses. However, it is still unclear how pathogens modulate host inflammatory responses. We found that a *Shigella* OspI effector delivered via the type III secretion system dampens acute inflammatory responses during bacterial invasion by targeting TNF receptor-associated factor 6 (TRAF6). OspI was a glutamine deamidase and selectively deamidated Gln100 to Glu100 in Ubc13. Consequently, the E2 ubiquitin-conjugating activity that is required for TRAF6 activation was inhibited, allowing *Shigella* OspI to modulate the diacylglycerol-CBM complex-TRAF6-NF-κB signaling pathway. We determined the 2.0 Å crystal structure of OspI, which contains a putative Cys-His-Asp catalytic triad. A mutational analysis showed that this catalytic triad was essential for deamidation activity. Our results suggest that *Shigella* inhibits acute inflammatory responses at the initial stage of infection by targeting the Ubc13-TRAF6 complex.

2. Shigella Targets Epithelial Tricellular Junctionsand Uses a Noncanonical Clathrin-Dependent Endocytic Pathway to Spread Between Cells.

Makoto Fukumatsu, Michinaga Ogawa, Satoko Arakawa[1], Masato Suzuki, Kazuhisa Nakayama[2], Shigeomi Shimizu[1], Minsoo Kim, Hitomi Mimuro, and Chihiro Sasakawa: [1]Department of Pathological Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan [2]Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

Bacteria move between cells in the epithelium us-

ing a sequential pseudopodium-mediated process but the underlying mechanisms remain unclear. We show that during cell-to-cell movement, Shigellacontaining pseudopodia target epithelial tricellularjunctions, the contact point where three epithelial cells meet. The bacteria-containing pseudopodia were engulfed by neighboring cells only in the presence of tricellulin, a protein essential for tricellular junction integrity. Shigella cell-to-cell spread, but not pseudopodium protrusion, also depended on phosphoinositide 3-kinase, clathrin, Epsin-1, and Dynamin-2, which localized beneath the plasma membrane of the engulfing cell. Depleting tricellulin, Epsin-1, clathrin, or Dynamin-2 expression reduced Shigella cell-to-cell spread, whereas AP-2, Dab2, and Eps15 were not critical for this process. Our findings highlight a mechanism for Shigella dissemination into neighboring cells via targeting of tricellular junctions and a noncanonical clathrin-dependent endocytic pathway.

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